

## The Sporadic Change of Epiblast Cells to Epithelial in the Chick Embryo before the Formation of Hensen's Knot

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**ABSTRACT**—33 chick embryos from stage 2 to stage 4 according to Hamburger and Hamilton [1] were stained *in toto* with Rhodamine phalloidin (Rh-P) and then surveyed with a fluorescence microscope.

Two types of cells were stained: one, elongated and stained along a whole cell surface, another, not elongated, stained eminently along the polygonal cell border at the upper surface. The former, recognized at or along the growing primitive streak (PS), were considered as the mesodermal cells because of their flask-like shape and downward inclination. The latter, appearing at the later stage of PS formation, were considered as the epithelial cells because of the polygonal frame work of F-actin along the cell border. At first, a few epithelial cells clustered to form small islands scattering along and slightly apart from the PS. The islands then increased in both number and size, becoming connected to each other forming two belts along the PS. When Hensen's knot was formed, all epiblastic cells differentiated into epithelial cells with the polygonal framework of F-actin.

### INTRODUCTION

The embryos of sea urchins, insects, fishes, or amphibians start morphogenetic movement only after the outer cell layer is established as a coherent cell sheet. The process during which the embryonic blastomeres were organized as an epithelium was precisely studied in the embryo of a fish, *Fundulus heteroclitus* [2]. At first, primitive zonula adherens was formed in the cells of the enveloping layer at the start of epiboly, which then developed to form a complete set of junctions during epiboly. At the initial stage of epiboly the microfilament bundles were observed to associate with zonula adherens scarcely but later more densely as like as those in the fully developed epithelial cells.

In the chick embryos, a 'specialized junction' was reported to be formed in the cells at margin of blastoderm during epiboly [3]. However, it is still not yet how or when the epiblast cells of area pellucida are organized to be an epithelium. To make clear of the period and the position when

and where the epithelial cells appeared, we made use the microfilament bundles associating with zonula adherens as a merkmal of epithelial differentiation. In the epithelial cells, F-actin, one of the main elements of the microfilament bundles can be recognized aligned polygonally along cell borders when stained with Rhodamine phalloidin (Rh-P). The appearance of polygonally aligned F-actin bundles in the embryonic cells could thus be considered as heralding the initiation of epithelial differentiation.

The chick embryos at various stages from the start of primitive streak (PS) formation to the establishment of Hensen's knot (HN), were stained *in toto* with Rh-P. At stage 3, according to Hamburger and Hamilton's (H and H) table [1], when PS is under development in the embryo, a small group of cells with the polygonally aligned F-actin bundles was recognized scattered along the primitive fold. As the PS grew, they increased in number, as did the number of cells within each group. The islands then fused together to make two belts running along the PS. By the time of HN formation, each cell of epiblast at the area pellucida came to have polygonal cell borders, suggesting that epithelial differentiation was completed.

This communication will present the details of observation and discussion on epithelial differentiation.

## MATERIALS AND METHODS

White leghorn eggs obtained commercially were incubated at 38°C for a required period.

The embryos were removed from both vitelline membrane and yolk in Tyrode solution, and staged according to the table below in which the stages determined by H and H were actually subdivided.

Stage 2: defined by H and H.

St. 2+: the embryo had PS length ranging from 500  $\mu\text{m}$  to 800  $\mu\text{m}$  (Fig. 2a).

St. 3—: the length of PS, ranged from 800 to 1500  $\mu\text{m}$  (Fig. 3a).

St. 3: defined by H and H. The embryo had PS longer than 1500  $\mu\text{m}$ , but without the discernible primitive groove.

St. 3+: with the definitive PS longer than 1500  $\mu\text{m}$ .

St. 4—: with the definitive PS, the anteriormost part of which was being surrounded by a circular fold.

St. 4: HN has been established.

1) Fixation Each embryo, the hypoblast of which was or was not removed, was mounted flat on a small piece of filter paper in Tyrode solution and put into a fixative (3.5% neutral formalin, 0.05% Triton X-100 in phosphate buffered saline (PBS) supplemented with 1 mM  $\text{MgCl}_2$  and 0.1 mM  $\text{CaCl}_2$ ) for 20 min at room temperature (RT, 20–25°C).

2) Staining After fixation, each sample was washed three times in PBS with the sheet of filter paper to which the sample was attached, and immersed in three drops of Rh-P solution (3 units/ml PBS, Wako Pure Chem. Co.) placed on the bottom of a plastic dish for 1 hr at RT in a dark, moist chamber.

Some of the samples were treated primarily with anti-actin antibody of rabbit (ICN, U.S.A.) diluted in PBS (1:10) for 1 hr at 38°C in the moist chamber. After being rinsed twice in PBS, they were stained with fluorescein isothiocyanate (FITC) conjugated anti-rabbit IgG antiserum of

goat (Cappel, U.S.A.) diluted in PBS (1:64) for 1 hr at 38°C in the moist chamber.

After stained with Rh-P or anti-actin antibody, each embryo attached to the filter paper was rinsed twice in PBS, separated from the paper, mounted on a slide with a drop of auto-fluorescence-free glycerine alkalinated with NaOH (pH=9) and supplemented with triethylendiamine (100 mg/ml), and coverglassed. The embryos were placed so that the outside of the epiblast cells faced the objective lenses. In order to protect the embryo from crushing with the cover glass, strips of filter paper were inserted between cover and slide.

Embryos with or without hypoblast were similarly stained.

3) Observation A microscope equipped with epifluorescent apparatus and a phase-contrast microscope were used. For microphotography, Tri-X pan negative films (Kodak) were used, which were processed with Superprodol (Fuji film Co.) for 8 min at 20°C to enhance the sensitivity of films from ISO 400 to 800.

## RESULTS

In a total, 33 embryos were successfully stained with Rh-P; 2 embryos at St. 2, 2, at St. 2+, 3, at St. 3—, 8, at St. 3, 3, at St. 3+, 8, at St. 4— and 4, at St. 4. 5 embryos (2, at St. 3 and 3, at St. 4) were stained with an indirect immunofluorescence method using anti-actin antibody as a first ligand. When stained with anti-actin, every cell in the embryo emitted dull fluorescence from the entire cell body and no discernible polygonal localization of F-actin was recognized throughout the stages observed.

However, when stained with Rh-P, two kinds of cells were identified and, furthermore, the stage-specific distribution pattern was observed.

1. *The embryos at St. 2.* With the phase contrast microscope, a small bulge of primitive streak (150  $\mu\text{m}$  or more in length) was recognized at the middle of the posterior half of area pellucida, very few cells of which were stained with Rh-P. A line of small dots of fluorescence was observed along the midline of PS (Fig. 1a). When focused just under the dots, sometimes, elongated cells were recognized which were stained weakly along the

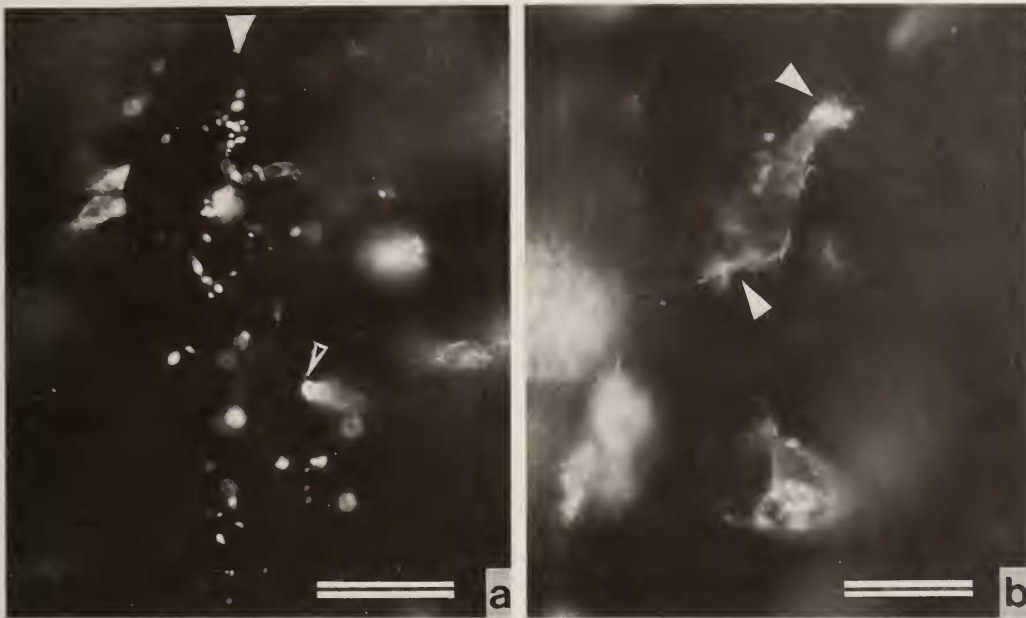


FIG. 1. The embryo at St. 2 stained *in toto* with Rh-P.

a The cells along PS. The white arrowhead shows the anteriormost part of PS, and the blank arrowhead, the part of cell left at the surface. The main body of cell elongated inwards. Bar, 50  $\mu$ m.

b A cell extending downwards. The upper arrowhead indicates the part of cell at the surface, the lower arrowhead, the invaginated cell body. Bar, 25  $\mu$ m.

cell surface but strongly at the end of cell body left at the epiblastic surface (Fig. 1b). Because of their elongated shapes and inward orientation, they were taken to be mesodermal cells invaginating to the interspace between epiblast and hypoblast.

2. *The embryos at St. 2+.* A number of small dots of fluorescence was observed along the whole extent of PS (Figs. 2a and 2b). Among them, a few small polygonal shapes, varying in size, were scattered along PS (Fig. 2c). Under the polygons, the elongated body of cells was recognized (cf. Fig. 1b), which were stained with Rh-P along their entire surface. Flask-like cells, though not so many, were also stained along the cell surface, the tails of which were, perhaps because of convergence of cell membrane, stained strongly in the form of white dots (Fig. 2c).

It is worth noting that, without exception, the flask cells elongated perpendicularly to PS (Fig. 2b).

3. *The embryo at the stages from 3- to 3.* Cells with small polygons of F-actin bundles at

the top were still observed along the midline of PS among many small dots (cf. Fig. 2c), the size of surface area of which varied from cell to cell. The extremely small ones reduced to be the end of a tail, the white dots. This suggests that the number of dots of fluorescence along PS are remnants of the mesodermal cells, which once had been polygonal in shape at epiblast and invaginated perpendicularly to PS (Fig. 3b).

Slightly apart from PS, polygonal cells (Fig. 4a) clustered into small islands. The cells did not elongate and instead, formed cellular protrusions at the bottom to contact with each other (Fig. 4b).

Focused on the hypoblast, a number of polygonal cells was scattered in solitary forms without making large clusters (cf. Fig. 3b).

4. *The embryo at stages from 3+ to 4-.* PS now grew to its maximum length. Many small dots of Rh-P were still observed at the bottom of the primitive groove. Within the primitive folds, a number of flask cells elongated perpendicularly to the groove.



The island of polygonal cells formed at St. 3, increased both in size and in number and, at St. 4—, fused together to make a belt of polygonal cells parallel to PS (Figs. 4c and 4d). At St. 4—, another group of polygonal cells were recognized, far from PS, along the outer border of area pellucida (Fig. 4c). Between these two areas, no cell had a polygonal framework.

Carefully observed, the polygons in embryonic cells were far from those in a fully developed epithelium [4]: their side lines in the early embryo were in imbalance and slack (Fig. 4d).

5. *The embryo at St. 4* In all embryos at this stage, each cell acquired polygonal fluorescence and stuck together. An entire span of area pellucida looked as if it was covered with a lacelike network of fluorescence (Fig. 5a). The bottom of the primitive groove was also covered with coherent cells, rather thin in their polygonal pattern (Fig. 5b). However, at the bottom of HN (Fig. 5c), small dots were still recognized, suggesting that invagination of cells was still continuing.

Despite our efforts, we could not obtain embryos in a transient state from St. 4— to St. 4, which might have shown us how the epiblast cells outside of the epithelial belts differentiated to

epithelial. Absence of the link may mean the rest of the epiblast cells differentiated in a short space of time (may be within 1 hr) once the epithelial belts were established.

## DISCUSSION

(1) *Staining a whole embryo with Rh-P.* NBD-phalloidin or Rh-P has been proved to be a good tool for surveying intracellular localization of F-actin bundles [5]. In this series of experiments, we applied Rh-P to whole embryos and observed the distribution pattern of cells stained with it. One of the merits of this method was that we could easily identify the place and the stage when a small number of cells appeared, even though they contained a small amount of F-actin bundles. With immunohistochemistry on sectioned samples or with electronmicrography, sparsely distributed cells laden with a small amount of F-actin cannot be detected so easily. The sections of embryonic cells are too thin for small accumulation of F-actin in the initial phase to be recognized. Although an electron microscope could identify F-actin deposited scarcely, its observation field is too small to cover a whole span

FIG. 2. The embryo at St. 2+.

- a The primitive streak observed with a phase contrast microscope. The upper arrowhead shows the anterior most part of PS, the under, the posterior most. Bar, 100  $\mu$ m.
- b The total figure of *Area pellucida* stained with Ph-P. White dots make a line overlapping the midline of PS. Note cells either right, or left, of the line elongated perpendicularly to it. White flakes at the background are fluorescence of cells in hypoblast. Bar, 100  $\mu$ m.
- c A magnified view of PS. Besides the white dots, polygons scatter. Bar, 50  $\mu$ m.
- d A magnified view of flask cells. The left arrowhead indicates a part of cell at the surface and the right, rest of the cells under the surface. Bar, 20  $\mu$ m.

FIG. 3. The embryo at St. 3—.

- a A total view, observed with a phase-contrast microscope. The upper arrowhead shows the anterior most part of PS, the under, the posterior most. Bar, 500  $\mu$ m.
- b A total view, observed with an epifluorescence microscope. The white arrowheads indicate both ends of PS. At the hemicycle area prior to the anterior end of PS, the cells are crowded but not coherent. White dots make a line along PS. A number of cells at both sides of PS elongates perpendicularly to PS along its entire length. Fluorescence at the back ground are of the cells in hypoblast. Bar, 250  $\mu$ m.

FIG. 4. The appearance of epithelial cells.

- a The islands of polygonal cells (St. 3+). The arrowhead shows the location of PS. Bar, 100  $\mu$ m.
- b The polygonal cells at St. 3—. Under the surface, the cells extend cellular protrusions (arrowheads) to contact with each other. Bar, 10  $\mu$ m.
- c At St. 4—, two belts consisting of polygonal cells run at both side of PS. The arrowhead indicates the anterior most of PS, where Hensen's knot is beginning to be formed. Note, at the area left, apart from the belt, another cluster of polygonal cells is shown. Bar, 100  $\mu$ m.
- d A magnified view of Fig. 4c. Note, each cell are bordered with rather irregular curved lines. The regular pentagons or hexagons are hardly observed. Bar, 50  $\mu$ m.



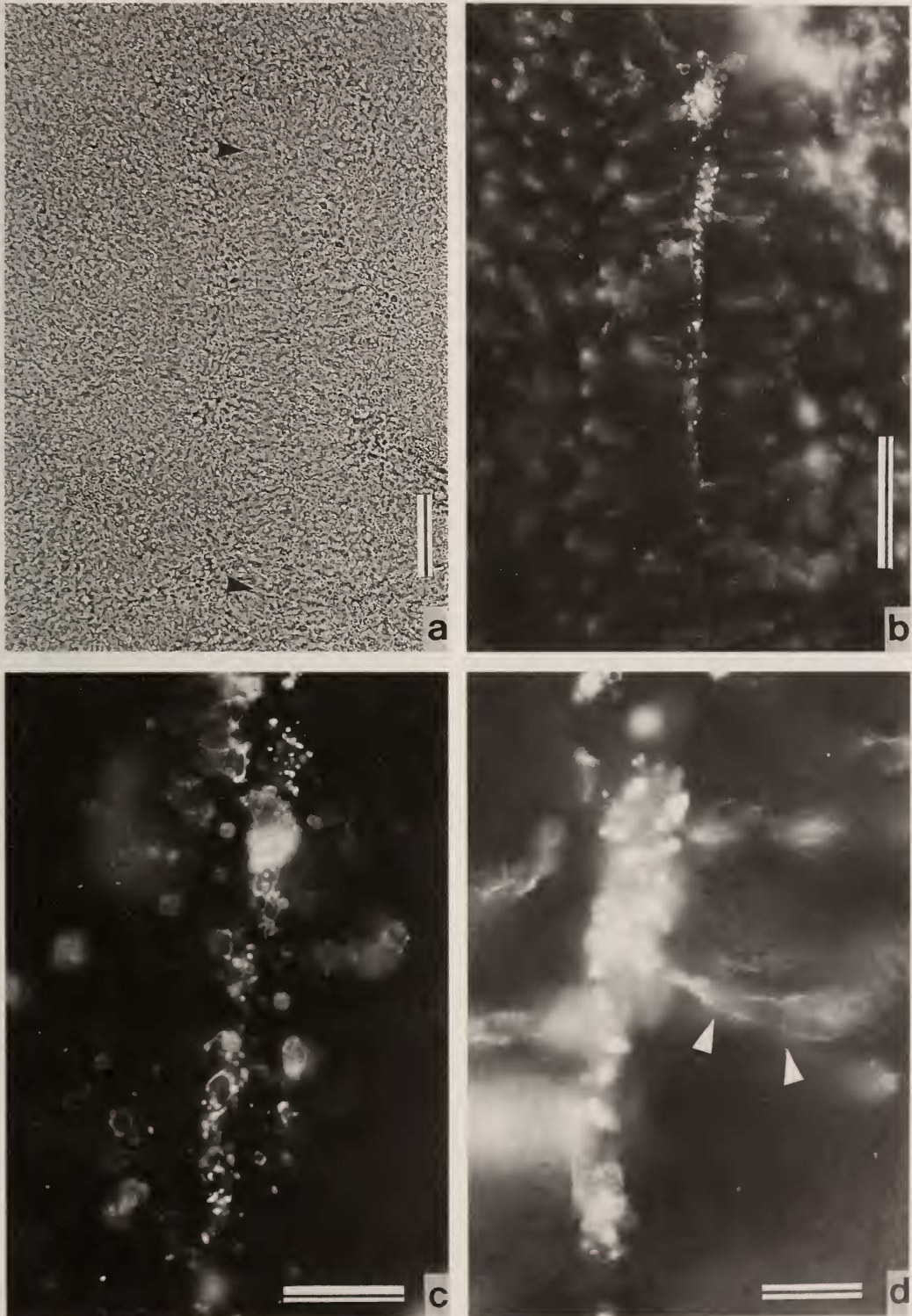


FIG. 2.

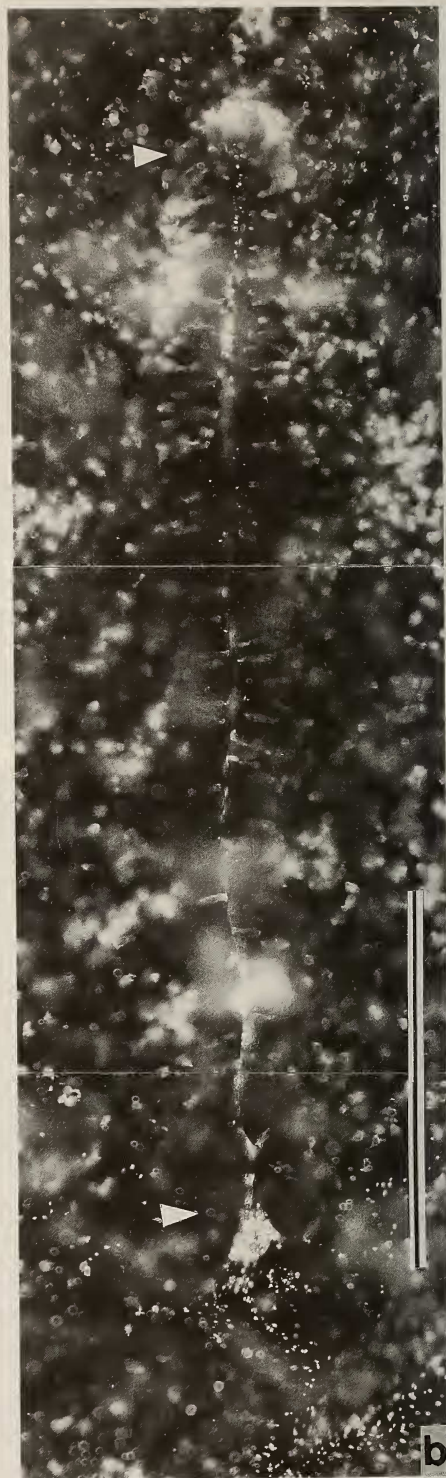
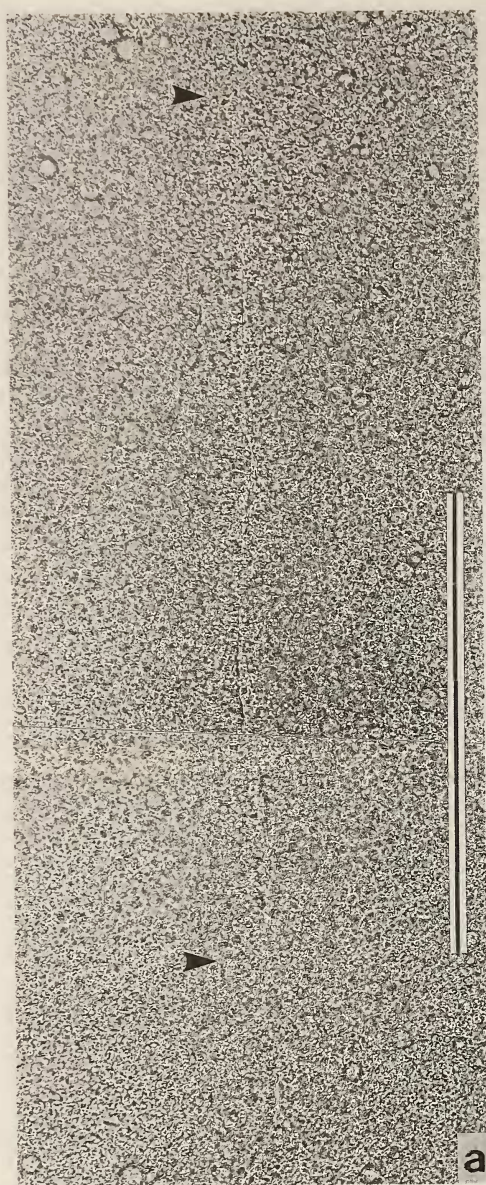


FIG. 3.



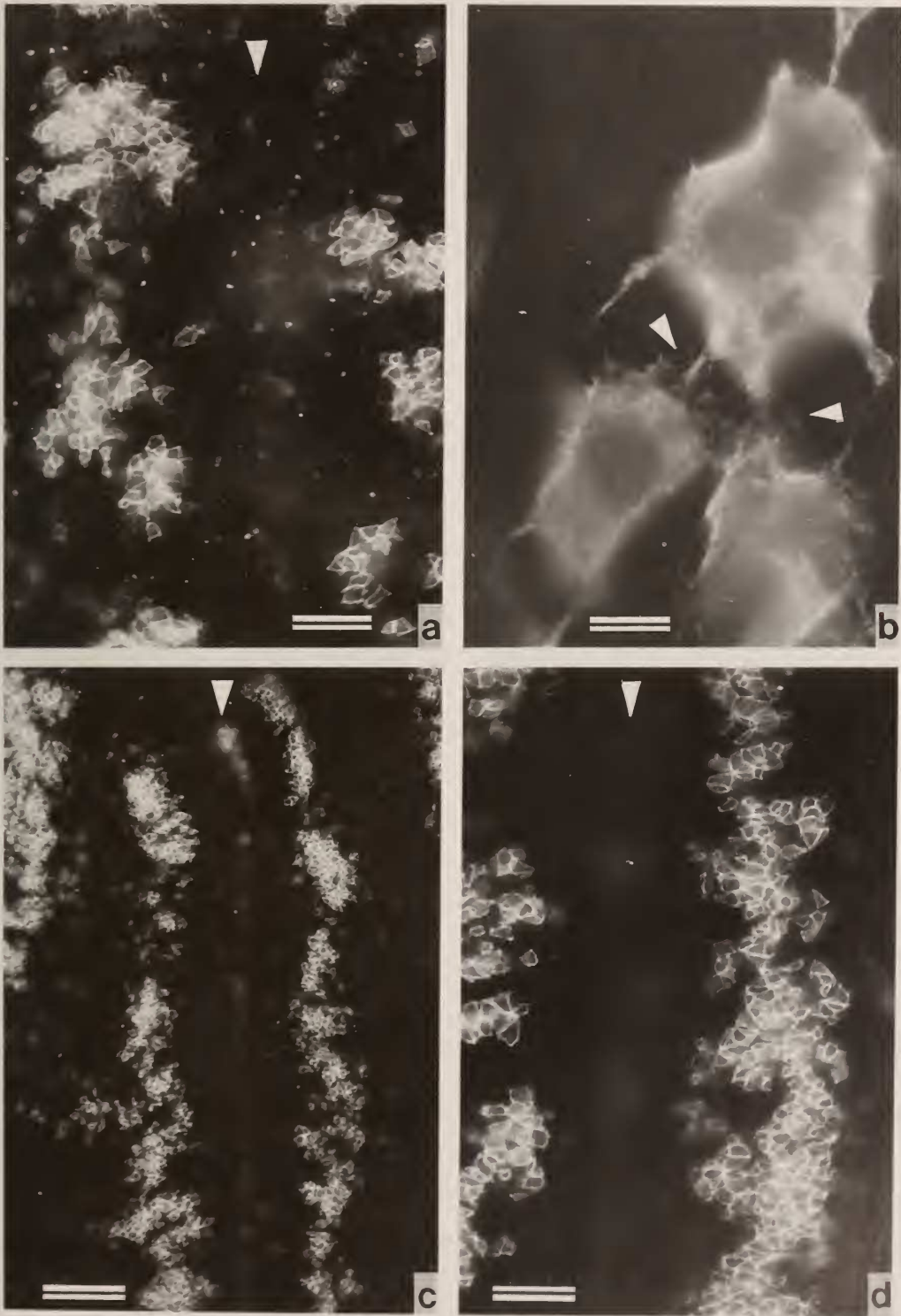


FIG. 4.



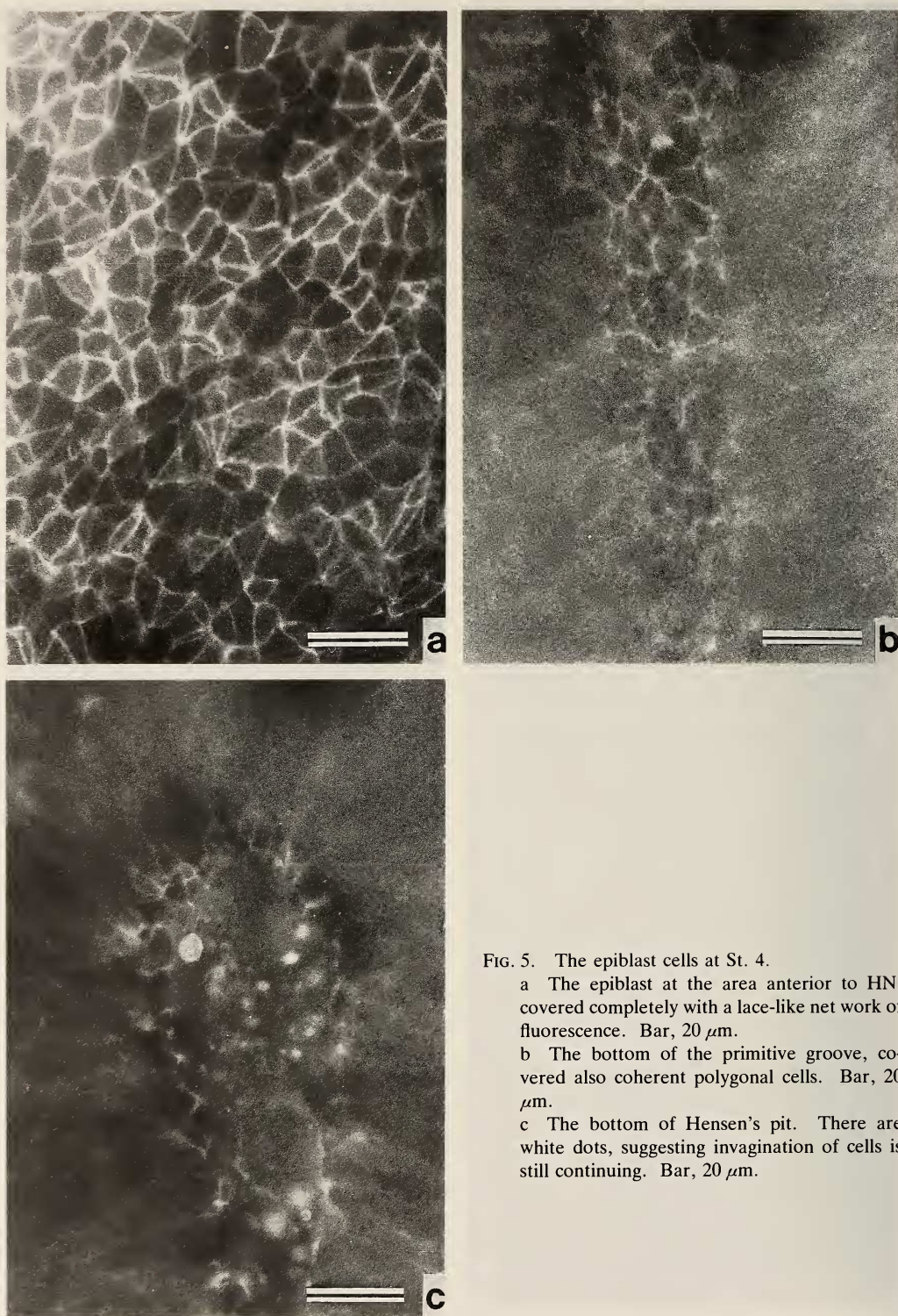


FIG. 5. The epiblast cells at St. 4.

a The epiblast at the area anterior to HN, covered completely with a lace-like net work of fluorescence. Bar, 20  $\mu$ m.

b The bottom of the primitive groove, covered also coherent polygonal cells. Bar, 20  $\mu$ m.

c The bottom of Hensen's pit. There are white dots, suggesting invagination of cells is still continuing. Bar, 20  $\mu$ m.

of area pellucida where the cells laden with F-actin were scattered.

Most of the cells at St. 2 showed no sign of fluorescence of Rh-P but emitted fluorescence of FITC, suggesting the presence of actin molecules but not in a polymerized form. There may be another explanation for this: F-actin in the embryo at a very early stage is different in nature from that at later stages that neither antibody against adult actin, nor Rh-P, bound enough to be recognized. However, it is reasonable for us to simply suppose that the cells at St. 2 accumulated insufficient F-actin to be observed with a light microscope.

(2) *The cell stained with Rh-P.* After St. 2, the cells stained with Rh-P were classified into two types: one was the elongated, solitary cell, along the whole cell surface of which was stained, and another, coherent, which were stained polygonally along cell borders.

The former including flask cells, were observed along PS in the embryos from St. 2 to St. 4—. They elongated perpendicularly to PS and sank obliquely. That is to say, their heads elongated inwards, with the tail remaining at the surface. Due to this inclination, they could be mesodermal cells invaginating. Elongation and a solitary form were confirmed as being specific to mesodermal cells: when fragments of presumptive mesoderm were cultured *in vitro*, the cells at the margin elongated enormously and exuded from the explant to wander about in a solitary form [6]. F-actin distributed along a cell surface is considered as perhaps generating the force for invagination. Having no evidence at hand, further discussion on this mechanism should be deterred.

The latter, considered epithelial, were recognized after St. 3—. In preliminary observation with a TEM, before St. 3—, the junction among epiblastic cells was primitive, consisting of only single bulges facing each other. After this stage, it was at the vicinity of these bulges that electron dense material was accumulated into an amorphous mass. Here, a complicated junction gradually developed downwards. If the electron dense material would be confirmed as F-actin, it would provide good support to our thesis that the epithelial cells laden with polygonal bundles of F-actin started to differentiate at stage 3.

Randomness in the area of epithelial differentiation suggests there was no dominant center coordinating the epithelial differentiation in area pellucida. However, there was a tendency for the cells along PS or HN to have differentiated earlier than those at other areas. This may be interpreted as follows: both intercellular and intracellular structures must have been strengthened in order to keep up the integrity of epiblast against the mechanical tension brought about by the actively invaginating cells. As a result, junctional complex and cytoskeletal structure developed quickly in cells characteristic of epithelial cells. Thus, epithelial differentiation occurred earlier at the area exposed to larger tension than that at less.

(3) *Irregularity of polygons in epithelialized epiblast.* The polygons observed in epiblastic cells were irregular and not well balanced in comparison with those in a typical epithelium showing a geometrical bee-hive pattern. For example, the corneal epithelium of an 8-day chick embryo consisted of well balanced pentagonal or septagonal cells stuck together, while the epiblast at St. 4 consisted of cells with slack side lines, the balance of which was lost (Fig. 5a).

This distortion may reflect the unstable state of the cells under morphogenetic movement. At wound healing, or in culture when the epithelial cells started to spread, even fully developed epithelial cells began to lose their geometrical regularity having slack side lines and lack of well-balanced proportion [4]. The mesodermal cells are invaginating actively through PS and HN. That means the continuity of the epiblast sheet is broken or, the epiblast has a wound in the middle. If the epiblast cells behaved so as to recover the continuity, the irregularity in cell shapes would be brought about as in the same way as corneal epithelial cells act during wound healing.

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